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(54) Title: PHYTASE COMBINATIONS			
(57) Abstract <p>The present invention relates generally to the use of at least two phytases of different position specificity, i.e. any combinations of 1-, 2-, 3-, 4-, 5-, and 6-phytases. By combining phytases of different position specificity a synergistic effect is obtained. Compositions such as food and feed or food and feed additives comprising such phytases in combination are also disclosed as are processes for their preparation.</p>			

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PHYTASE COMBINATIONS

TECHNICAL FIELD

The present invention relates generally to the use of at least two phytases of different
5 position specificity. In particular, the invention relates to animal feed and animal feed
additives comprising such phytases.

BACKGROUND ART

Phytic acid or myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (or for short myo-
10 inositol hexakisphosphate) is the primary source of inositol and the primary storage
form of phosphate in plant seeds. In fact, it is naturally formed during the maturation of
seeds and cereal grains. In the seeds of legumes it accounts for about 70% of the
phosphate content and is structurally integrated with the protein bodies as phytin, a
mixed potassium, magnesium and calcium salt of inositol. Seeds, cereal grains and
15 legumes are important components of food and feed preparations, in particular of
animal feed preparations. But also in human food cereals and legumes are becoming
increasingly important.

The phosphate moieties of phytic acid chelates divalent and trivalent cations such as
20 metal ions, i.e. the nutritionally essential ions of calcium, iron, zinc and magnesium as
well as the trace minerals manganese, copper and molybdenum.

Besides, the phytic acid also to a certain extent binds proteins, including digestive
enzymes, by electrostatic interaction. At a pH below the isoelectric point, pI, of the
25 protein, the positively charged protein binds directly with phytate. At a pH above pI, the
negatively charged protein binds via metal ions to phytate.

Phytic acid and its salts, phytates, are often not metabolized, since they are not
absorbable from the gastro intestinal system, i.e. neither the phosphorous thereof, nor
30 the chelated metal ions, nor the bound proteins are nutritionally available.

Accordingly, since phosphorus is an essential element for the growth of all organisms, food and feed preparations need to be supplemented with inorganic phosphate. Quite often also the nutritionally essential ions such as iron and calcium, must be supplemented. And, besides, the nutritional value of a given diet decreases, because of
5 the binding of proteins by phytic acid. Accordingly, phytic acid is often termed an anti-nutritional factor.

Still further, since phytic acid is not metabolized, the phytate phosphorus passes through the gastrointestinal tract of such animals and is excreted with the manure,
10 resulting in an undesirable phosphate pollution of the environment resulting e.g. in eutrophication of the water environment and extensive growth of algae.

Phytic acid or phytates, said terms being, unless otherwise indicated, in the present context used synonymously or at random, are degradable by phytases.

15

The production of phytases by plants as well as by microorganisms has been reported. Amongst the microorganisms, phytase producing bacteria as well as phytase producing fungi are known.

20 From the *plant* kingdom, phytases of e.g. wheat-bran and maize root have been described (Thomlinson et al, Biochemistry, 1 (1962), 166-171; and Hübel, F. & E. Beck; Plant Physiology (1996), 112: 1429-1436, respectively). An alkaline phytase from lilly pollen has been described by Barrientos et al, Plant. Physiol., 106 (1994), 1489-1495. These plant phytases are generally formed during the germination of the
25 seed and serve the purpose of liberating phosphate and, as the final product, free myo-inositol for use during the plant growth.

Amongst the *bacteria*, phytases have been described which are derived from *Bacillus subtilis* (Paver and Jagannathan, 1982, *Journal of Bacteriology* 151:1102-1108) and
30 *Pseudomonas* (Cosgrove, 1970, *Australian Journal of Biological Sciences* 23:1207-1220). Still further, a phytase from *E. coli* has been purified and characterized by

Greiner et al, Arch. Biochem. Biophys., 303, 107-113, 1993). However, this enzyme is probably an acid phosphatase.

Phytase producing yeasts are also described, such as *Saccharomyces cerevisiae* (Nayini et al, 1984, *Lebensmittel Wissenschaft und Technologie* 17:24-26. However, this enzyme is probably a myo-inositol monophosphatase (Wodzinski et al, Adv. Appl. Microbiol., 42, 263-303). AU-A-24840/95 describes the cloning and expression of a phytase of the yeast *Schwanniomyces occidentalis*.

There are several descriptions of phytase producing filamentous fungi. In particular, there are several references to phytase producing ascomycetes of the *Aspergillus* genus such as *Aspergillus terreus* (Yamada et al., 1986, *Agric. Biol. Chem.* 322:1275-1282). Also, the cloning and expression of the phytase gene from *Aspergillus niger* var. *awamori* has been described (Piddington et al., 1993, *Gene* 133:55-62). EP 0 420 358 describes the cloning and expression of a phytase of *Aspergillus ficuum* (*niger*). EP 0 684 313 describes the cloning and expression of phytases of the ascomycetes *Myceliophthora thermophila* and *Aspergillus terreus*.

Nomenclature and position specificity of phytases

In the present context a phytase is an enzyme which catalyzes the hydrolysis of phytate (myo-inositol hexakisphosphate) to (1) myo-inositol and/or (2) mono-, di-, tri-, tetra- and/or penta-phosphates thereof and (3) inorganic phosphate. In the following, for short, the above compounds are sometimes referred to as IP6, I, IP1, IP2, IP3, IP4, IP5 and P, respectively. This means that by action of a phytase, IP6 is degraded into P + one or more of the components IP5, IP4, IP3, IP2, IP1 and I. Alternatively, myo-inositol carrying in total n phosphate groups attached to positions p, q, r,... is denoted Ins(p,q,r,...)Pn. For convenience Ins(1,2,3,4,5,6)P6 (phytic acid) is abbreviated PA.

According to the Enzyme nomenclature database ExPASy (a repository of information relative to the nomenclature of enzymes primarily based on the

recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) describing each type of characterized enzyme for which an EC (Enzyme Commission) number has been provided), two different types of phytases are known: A so-called 3-phytase (myo-inositol
5 hexaphosphate 3-phosphohydrolase, EC 3.1.3.8) and a so-called 6-phytase (myo-inositol hexaphosphate 6-phosphohydrolase, EC 3.1.3.26). The 3-phytase hydrolyses first the ester bond at the 3-position, whereas the 6-phytase hydrolyzes first the ester bond at the 6-position.

10 Inositolphosphate nomenclature

Considering the primary hydrolysis products of a phytase acting on phytic acid, i.e. the penta phosphate ester IP5 or Ins(p,q,r,-)P5, some of the resulting esters are diastereomers and some are enantiomers. Generally, it is easier to discriminate between diastereomers, since they have different physical properties, whereas it is
15 much more difficult to discriminate between enantiomers which are mirror images of each other.

Thus, Ins(1,2,4,5,6)P5 (3-phosphate removed) and Ins(1,2,3,4,5)P5 (6-phosphate removed) are diastereomers and easy to discriminate, whereas Ins(1,2,4,5,6)P5 (3-phosphate removed) and Ins(2,3,4,5,6)P5 (1-phosphate removed) are enantiomers.
20 The same holds true for the pair Ins(1,2,3,4,5)P5 (6-phosphate removed) and Ins(1,2,3,5,6)P5 (4-phosphate removed). Accordingly, of the 6 penta-phosphate esters resulting from the first step of the phytase catalyzed hydrolysis of phytic acid, you can only discriminate easily between those esters in which the 2-, 3-, 5- and 6-phosphate has been removed, i.e. you have four diastereomers only, each of the
25 remaining two esters being an enantiomer of one each of these compounds (4- and 6- are enantiomers, as are 1- and 3-).

Use of lowest-locant rule

30 It should be noted here, that when using the notations Ins(2,3,4,5,6)P5 and Ins(1,2,3,5,6)P5, a relaxation of the previous recommendations on the numbering of

the atoms of myo-inositol has been applied. This relaxation of the lowest-locant rule is recommended by the Nomenclature Committee of the International Union of Biochemistry (Biochem. J. (1989) 258, 1-2) whenever authors wish to bring out structural relationships.

5

In this lowest-locant rule, the L- and D-nomenclature is recommended: Inositolphosphate, phosphate esters of myo-inositol, are generally designated 1D- (or 1L-) -Ins(r,s,t,u,w,x)P_n, n indicating the number of phosphate groups and the locants r,s,t,u,w and x, their positions. The positions are numbered according to the
10 Nomenclature Committee of the International Union of Biochemistry (NCIUB) cited above (and the references herein), and 1D or 1L is used so as to make a substituent have the lowest possible locant or number ("lowest-locant rule").

Phytase specificity

15 As said above, phytases are divided according to their specificity in the initial hydrolysis step, viz. according to which phosphate-ester group is hydrolyzed first.

As regards the specificity of known phytases, plant phytases are generally said to be 6-phytases. However the lilly pollen phytase is said to be a 5-phytase. The
20 microorganism derived phytases are mainly said to be 3-phytases. E.g. the ExPASy database mentioned above refers for 3-phytases to four phytases of *Aspergillus awamori* (strain ALK0243) and *Aspergillus niger* (strain NRRL 3135) (Gene 133:55-62 (1993) and Gene 127:87-94 (1993)).

25 SUMMARY OF THE INVENTION

It has now been found that phytate phosphorous, when phytate is subjected to the combined action of two phytases of different position specificity, is released more efficiently from i.a. phytate. In other words a synergistic effect is seen by using two phytase enzymes with different modes of action. In this way phosphorous may be
30 released more quickly, and/or more phosphorous may be liberated from the substrate, as compared to a process where the substrate is treated with only one of

these phytases. Such an effect is of great importance in animal nutrition where in some species such as poultry or fish the digesta transit time is short thereby limiting the time available for an enzyme to act. In other species, such as the pig, the feed consumed has a long residence time in the acid environment in the stomach which
5 has a negative influence on the performance of any added enzyme. Consequently a rapid and more efficient removal of phosphorus groups from the phytate molecule is of similar importance in pigs.

Accordingly, in its first aspect, the invention provides compositions, which comprise at
10 least two phytases of different position specificity. Examples of such compositions are food and animal feed as well as additives for use in food and feed.

In other aspects, the invention provides processes for preparing these compositions, and the use of these compositions for various purposes.

15

In particular, the invention provides a method of enzymatic release of phosphorus from a phytase substrate, in particular a phytate containing substrate, which method comprises subjecting the substrate to the simultaneous action of a 3-phytase (EC 3.1.3.8) and a 6-phytase (EC 3.1.3.26).

20

DETAILED DISCLOSURE OF THE INVENTION

Generally, the invention, relates to the use of at least two phytases of different position specificity. Preferably, the at least two phytases are distinct or different enzymes.

25 The phytases are selected from amongst the group consisting of 2-phytases, 3-phytases, 5-phytases and 6-phytases (as defined below to include 1-phytase in the definition of 3-phytase and 4-phytase in the definition of 6-phytase).

Any sub-group selected from this group, which sub-group consists of any set of two,
30 any set of three or all four members of the group, is within the scope of this invention.

Preferred examples of sub-groups of two phytases are, using in this paragraph the notation "N" for "a N-phytase:" 3+6 (viz. a 3-phytase and a 6-phytase); 2+6; 5+6; 3+2; 3+5; 2+5.

5

Preferred examples of sub-groups of three phytases are, using in this paragraph also the notation "N" for "a N-phytase:" 2+5+6 (viz. a 2-phytase and a 5-phytase and a 6-phytase); 2+5+3; 3+6+2; 3+6+5; 6+2+3; 6+2+5.

- 10 A preferred example of a sub-group of four members is, using in this paragraph also the notation "N" for "a N-phytase:" 2+5+6+3.

The phytase activity can be determined using any assay in which a phytase substrate is used. Examples (non-exhaustive list) of phytase substrates are the various myo-
15 inositol phosphates. Phytic acid and any salt thereof, e.g. sodium phytate or potassium phytate or mixed salts, are preferred substrates. Also any stereoisomer of the mono-, di-, tri-, tetra- or penta-phosphates of myo-inositol might serve as a phytase substrate. In the present context (unless otherwise specified) the phytase activity is determined in the unit of FYT, one FYT being the amount of enzyme that
20 liberates 1 μmol inorganic ortho-phosphate per min. under the following conditions: pH 5.5; temperature 37°C; substrate: sodium phytate ($\text{C}_6\text{H}_6\text{O}_{24}\text{P}_6\text{Na}_{12}$) in a concentration of 0.0050 mol/l. A suitable phytase assay is described below.

The phytase specificity can be examined in several ways, e.g. by HPLC or by NMR
25 spectroscopy, as further outlined in the experimental part. These methods, however, do not immediately allow the discrimination between hydrolysis of e.g. the phosphate-ester groups in positions D-6 and L-6, since the products of the hydrolysis, D-Ins(1,2,3,4,5)P5 and L-Ins(1,2,3,4,5)P5, are enantiomers (mirror images), and therefore have identical NMR spectra.

30

Generally, in the present context a N-phytase (N = 2, 3, 5 or 6) means a phytase which hydrolyzes first the N-position in phytic acid or has a preference for this position / these positions (e.g. N=6 covers two positions, as further outlined below).

- 5 In particular, in the present context a 6-phytase means either of a L-6- or a D-6-phytase or both, viz. a phytase being a L-6-phytase, a D-6-phytase or a ((D-6-)+(L-6-))-phytase (having both activities). The latter is sometimes also designated a D/L-6-phytase.
- 10 Likewise, a 3-phytase means either of a D-3-, L-3- or D/L-3-phytase.

Using these definitions, the concept of a 4-phytase is comprised in the definition of a 6-phytase, and the concept of a 1-phytase is comprised in the definition of a 3-phytase. A 5-phytase and a 2-phytase, on the other hand, comprise only one type of
15 enzymes each, viz. those in which the penta phosphate ester resulting from the first step of the hydrolysis of phytic acid is unambiguously defined.

Using a 6-phytase preferably more than 50% of the hydrolysis product of the first step is Ins(1,2,3,4,5)P5 and/or Ins(1,2,3,5,6)P5. Preferably these two compounds
20 comprise at least 60%, more preferably at least 70%, still more preferably at least 80%, especially at least 90% and mostly preferred more than 95% of the product of the initial hydrolysis step of PA.

Using an N-phytase (N = 2, 3 or 5), preferably more than 50, 60, 70, 80, 90, or mostly
25 preferred more than 95% of the hydrolysis product of the first step is Ins(1,3,4,5,6)P5; (Ins(2,3,4,5,6)P5 and/or Ins(1,2,4,5,6)P5); or Ins(1,2,3,4,6)P5, respectively.

Corresponding preferred embodiments (more than 50, 60, 70, 80, 90, 95%) apply with respect to a D-6-; a L-6-; a D/L-6-; a D-3-; a L-3-; and a D/L-3-phytase.

Further preferred examples of sub-groups of two phytases are (using in this paragraph the notations "P" for "a P-phytase (P = 2 or 5) and "DN," "LN" or "D/LN" (N = 3 or 6) for "a D-N-phytase," a "L-N-phytase," or a "D/L-N-phytase," respectively) are: D6+L6; D3+L3; D6+D3; D6+L3; L6+D3; L6+L3; D6+2; D6+5; L6+2; L6+5; D3+2; 5 D3+5; D6+2; D6+5; L6+2; L6+5; D/L6+L6; D/L6+D6; D/L6+L3; D/L6+D3; D/L6+D/L3; D/L6+2; D/L6+5 etc.

Further preferred examples of sub-groups of three phytases are (using the same notation as in the paragraph above): D6+L6+D3; D6+L6+L3; D3+L3+D6; D3+L3+L6; 10 D6+D3+2; D6+D3+5; D6+L3+2; L6+L3+2; L6+D3+2; D6+L3+5; L6+L3+5; L6+D3+5; D/L6+L3+L6; D/L6+L3+D6; D/L6+L3+5; D/L6+D3+5; D/L6+D/L3+5; D/L6+D3+2; D/L6+L3+2 etc.

In a preferred embodiment the 3-phytase is of microbial origin. In a more preferred 15 embodiment, the 3-phytase is of fungal origin. In a most preferred embodiment, the 3-phytase is derived from a strain of *Aspergillus*, in particular from a strain of *Aspergillus awamori*, a strain of *Aspergillus ficuum*, or a strain of *Aspergillus niger*. A microbial 3-phytase, Phytase Novo™, derived from *Aspergillus niger*, is commercially available from Novo Nordisk A/S, Denmark.

20

In another preferred embodiment, the 6-phytase is of plant origin. In a more preferred embodiment, the 6-phytase is derived from a wheat, rye or maize plant. Wheat phytase is commercially available, e.g. from Sigma (P-1259).

25 In a yet more preferred embodiment the 6-phytase is of bacterial origin, preferably derived from a strain of *Escherichia coli*, in particular the strain *Escherichia coli* K12, ATCC 33965. A 6-phytase from the strain *Escherichia coli* K12, ATCC 33965 has been purified and characterized by Greiner et al. [Greiner R, Konietzny U and Jany KI-D; Archives of Biochemistry and Biophysics, 1993, 303 (1), 107-113].

In another preferred embodiment, the 6-phytase is of fungal origin. In particular derived from a fungal strain of the order Aphyllophorales, in particular of the family Corticiaceae, especially from the genus *Peniophora*. A most preferred 6-phytase is derived from *Peniophora lycii*, in particular *Peniophora lycii* CBS 686.96, or a mutant or a variant thereof. The isolation and purification of this phytase is described in Examples 1 and 2 below.

A preferred 5-phytase is derived from lilly pollen.

The phytases are preferably added in the form of mono-component preparations, i.e. 5 enzyme preparations in which for instance essentially all of the 3-phytase activity or the 6-phytase activity (the phytase activity detectable) is owing to single phytase components.

In a first aspect, the invention relates to compositions comprising at least two 10 phytases of different position specificity and processes for preparing such compositions.

In the present context a composition comprising at least two phytases of different position specificity means any natural or synthetic product which has been enriched 15 with such phytases. Although a composition comprising only a mixture of the at least two phytases (and nothing more) is also included in this definition, the composition usually also contains other components. Examples of such compositions are food and feed, as well as phytase preparations, in particular food and feed additives.

20 A feed and a food, respectively, means any natural or artificial diet, meal or the like or components of such meals intended or suitable for being eaten, taken in, digested, by an animal and a human being, respectively. Such product is prepared by mixing feed or food components, to usually provide a desired amount of protein, carbohydrates, lipids, vitamins and minerals. Optionally, also other steps are included in the process 25 for preparing food and feed, such as a heat treatment step.

Phytase preparations (formulated enzyme compositions or preparations) are generally liquid or dry.

- 5 Liquid preparations need not contain anything more than the phytase enzymes, preferably in a highly purified form. Usually, however, a stabilizer such as glycerol, sorbitol or mono propylen glycol is also added. The liquid preparation may also comprise other additives, such as salts, sugars, preservatives, pH-adjusting agents, proteins, phytate (a phytase substrate). Typical liquid preparations are aqueous or oil-
10 based slurries. The liquid preparations can be added to a food or feed after an optional pelleting thereof.

- Dry preparations may be spray dried preparations, in which case the preparation need not contain anything more than the enzymes in a dry form. Usually, however,
15 dry preparations are so-called granulates which may readily be mixed with e.g. food or feed components, or more preferably, form a component of a pre-mix. The particle size of the enzyme granulates preferably is compatible with that of the other components of the mixture. This provides a safe and convenient mean of incorporating enzymes into e.g. animal feed.

20

Agglomeration granulates are prepared using agglomeration technique in a high shear mixer (e.g. Lödige) during which a filler material and the enzyme are co-agglomerated to form granules. Absorption granulates are prepared by having cores of a carrier material to absorb/be coated by the enzyme.

25

Typical filler materials are salts such as disodium sulphate. Other fillers are kaolin, talc, magnesium aluminium silicate and cellulose fibres. Optionally, binders such as dextrins are also included in agglomeration granulates.

- 30 Typical carrier materials are starch, e.g. in the form of cassava, corn, potato, rice and wheat. Salts may also be used.

Optionally, the granulates are coated with a coating mixture. Such mixture comprises coating agents, preferably hydrophobic coating agents, such as hydrogenated palm oil and beef tallow, and if desired other additives, such as calcium carbonate or
5 kaolin.

Additionally, phytase preparations may contain other substituents such as coloring agents, aroma compounds, stabilizers, vitamins, minerals, other feed or food enhancing enzymes etc. This is so in particular for the so-called pre-mixes.

10

A food or feed additive is composed as indicated for phytase preparations above and intended for or suitable for being added to food or feed. Basically, such additive is prepared by mixing the additive components, examples of which are mentioned above and below. In particular it is a substance which by its intended use is becoming
15 a component of a food or feed product or affects any characteristics of a food or feed product. A typical additive usually comprises one or more compounds such as vitamins, minerals or feed enhancing enzymes and suitable carriers and/or excipients.

20 In a preferred embodiment, the phytase preparations or additives of the invention additionally comprise an effective amount of one or more feed enhancing enzymes, in particular feed enhancing enzymes selected from the group consisting of α -galactosidases, β -galactosidases, in particular lactases, other phytases, β -glucanases, in particular endo- β -4-glucanases and endo- β -1,3(4)-glucanases,
25 cellulases, xylosidases, galactanases, in particular arabinogalactan endo-1,4- β -galactosidases and arabinogalactan endo-1,3- β -galactosidases, endoglucanases, in particular endo-1,2- β -glucanase, endo-1,3- α -glucanase, and endo-1,3- β -glucanase, pectin degrading enzymes, in particular pectinases, pectinesterases, pectin lyases, polygalacturonases, arabinanases, rhamnogalacturonases, rhamnogalacturonan
30 acetyl esterases, rhamnogalacturonan- α -rhamnosidase, pectate lyases, and α -galacturonisidases, mannanases, β -mannosidases, mannan acetyl esterases, xylan

acetyl esterases, proteases, xylanases, arabinoxylanases and lipolytic enzymes such as lipases, phospholipases and cutinases.

The animal feed additive of the invention is supplemented to the animal before or
5 simultaneously with the diet. Preferably, the animal feed additive of the invention is supplemented to a mono-gastric animal simultaneously with the diet. In a more preferred embodiment, the animal feed additive is added to the diet in the form of a granulate or a stabilized liquid.

10 An effective overall amount of phytase in food or feed is from about 10-20.000; preferably from about 10 to 15.000, more preferably from about 10 to 10.000, in particular from about 100 to 5.000, especially from about 100 to about 2.000 FYT/kg feed or food.

15 The phytase may exert its effect in vitro or in vivo, i.e. before intake or in the stomach of the individual, respectively. Also a combined action is possible.

In a second aspect, the invention relates to the use of at least two phytases of different position specificity for liberating phosphorous from a phytase substrate.

20

Preferably, the two or more different types of phytase enzymes referred to above are active simultaneously. In another preferred embodiment, they act sequentially, i.e. one type is acting first, the second type subsequently etc. In the sequential type of action the first type may be active or non-active, when the second type exerts its effect etc.,
25 viz. a simultaneous and sequential, and a pure sequential action, respectively. In all these cases a synergistic effect is observed on the release of phosphorous from the phytase substrate, in particular inorganic phosphate from phytate. In a preferred embodiment the enzymes are active simultaneously.

The effect can be exerted *in vitro*, viz. e.g. by pre-treating animal feed, or *in vivo*, i.e. in the digestive system of the animal. In animal feed, the phytases are preferably active *in vivo*.

- 5 This use can be performed on any composition which comprises at least one phytase substrate; i.e. at least one substrate towards which the phytases show an activity.

This composition may be an essentially pure mono component substrate or - which is usually the case - based on or originating from a complex biological material, such as
10 cereals and legumes, in particular seeds and cereal grains, e.g. seeds of legumes.

Examples (non-exhaustive list) of phytase substrates are various myo-inositol phosphates. Phytic acid and any salt thereof, e.g. sodium phytate or potassium phytate or mixed salts, are preferred substrates. Also any stereoisomer of the mono-,
15 di-, tri-, tetra- or penta-phosphates of myo-inositol might serve as a phytase substrate.

Preferably, the phytase substrate is present in amounts significant for being a source of phosphorous. More preferably, the phytase substrate comprises phytate (as broadly defined previously to include phytic acid and phytin).

A preferred composition is an animal feed composition comprising soy bean, field beans, peas, lupines, linseed, sunflower, rape, or cereals, in particular barley, wheat, oat, corn, sorghum, etc. Usually, these animal feed compositions are intended for mono-gastric animals. In the context of this invention, mono-gastric animals include i.a. fish, poultry, in particular broiler chicks, layers and turkeys, pigs, in particular piglets, and young calves.

When using the phytases, "phosphorous" is released. The term "phosphorous" is to be interpreted broadly, e.g. it is intended to include i.a. inorganic and organic bound

phosphorous, whatever the form, e.g. inorganic ortho phosphate, and any P-comprising substituents.

The use of the invention may be carried out at conditions usually employed for
5 release of phosphorous from a phytase substrate, e.g. phytate. Of course, generally, the conditions are chosen balancing on one hand the optimum conditions of the two enzymes in question, and on the other hand the optimum conditions for the particular application. Some examples of such conditions which could be relevant in practice are described below.

10

In particular the pH may be in the range of from about pH 2 to about pH 8, preferably of from about 3 to about 7, in particular of from about 2 to about 6, still more preferably of from about 4 to about 6. The temperature may be in the range of from about 20°C to about 80°C, preferably of from about 20°C to about 60°C, still more
15 preferably 30-60°C, alternatively around 37-58°C. The reaction time may be of from about 15-30 minutes to about 24 hours, preferably of from about 30 minutes to 6 hours, still more preferably 30 minutes to 4 hours; 30 minutes to 3 hours, 30 minutes to 2 hours, even more preferably 30 minutes to 1 hour. Also a reaction time of from about 2 to about 8 hours is highly relevant in feed for pigs.

20

An effective overall amount of phytase in food or feed is from about 10-20.000; preferably from about 10 to 15.000, more preferably from about 10 to 10.000, in particular from about 100 to 5.000, especially from about 100 to about 2.000 FYT/kg feed or food. Usually, animal feed has a dry matter content of 10-20%.

25

In a preferred embodiment the animal feed additive comprises phytase enzymes in total amounts corresponding to of from about 200 to about 50,000 Phytase Units (FYT) per gram of the composition, preferably of from about 500 to about 10,000 FYT per gram of the composition, more preferably of from about 2000 to about 6000 FYT per
30 gram of the composition, most preferably of from about 2500 to about 5000 FYT/g.

EXAMPLE 1**Purification of the phytase of *Peniophora lycii* CBS 686.96**Materials and MethodsMedia:5 *Phytate replication plates:*

Add to 200ml of melted SC agar

20ml 20% galactose

800µl 5% threonine

25ml solution A

10 25ml solution B

200µl Trace element solution (DSM Catalogue 141)

*Solution A:*6g CaCl₂, 2H₂O15 8g MgCl₂, 6H₂Oadd ddH₂O to 1l

pH = 6.5

Solution B:

20 35.12g Na-phytate

add H₂O to 1l

pH = 6.5

Medium A:

25	Yeast Nitrogen Base w/o Amino acids (Difco0919)	7.5 g/l
	Succinic acid (Merck 822260)	11.3 g/l
	NaOH (Merck 6498)	6.8 g/l
	Casamino acid w/o vitamin (Difco 0288)	5.6 g/l
	tryptophan (Merck 8374)	0.1 g/l
30	Threonine	1.0 g/l
	Na-phytate (35.12 g/l pH 6.5)	125 ml/l

	Galactose	20.0 g/l
	Trace metal (DSM 141)	1.0 ml/l
	ad 1l with H ₂ O	
5	<i>Trace metal solution:</i>	
	Nitrilotriacetic acid	1.50 g
	MgSO ₄ ·7 H ₂ O	3.00 g
	MnSO ₄ ·2H ₂ O	0.50 g
	NaCl	1.00 g
10	FeSO ₄ ·7H ₂ O	0.10 g
	CoSO ₄ ·7H ₂ O	0.18 g
	CaCl ₂ ·2H ₂ O	0.10 g
	ZnSO ₄ ·7H ₂ O	0.18 g
	CuSO ₄ ·5H ₂ O	0.01 g
15	KAl(SO ₄) ₂ ·12H ₂ O	0.02 g
	H ₃ BO ₃	0.01 g
	Na ₂ MoO ₄ ·2H ₂ O	0.01 g
	NiCl ₂ ·6H ₂ O	0.025 g
	Na ₂ Se ₃ O ₅ ·5H ₂ O	0.30 g
20	Distilled water	1 l
	pH 7.0	

First dissolve nitrilotriacetic acid and adjust pH to 6.5 with KOH, then add minerals.
Final pH 7.0 (with KOH).

25

Medium B:

Similar to medium A except for glucose is added as a C-source instead of galactose.

YPD:

- 30 10 g yeast extract, 20 g peptone, H₂O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM:

10 g yeast extract, 20 g peptone, H₂O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

5

10 x Basal salt:

75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H₂O ad 1000 ml, sterile filtered.

10 *SC-URA:*

100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H₂O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

15 *SC-agar:*

SC-URA, 20 g/l agar added.

SC-variant agar:

20 g agar, 20 ml 10 x Basal salt, H₂O ad 900 ml, autoclaved

20

General molecular biology methods

Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Unless otherwise specified all enzymes for DNA manipulations, such as e.g. restriction endonucleases, ligases etc., were obtained from New England Biolabs, Inc. The enzymes were used according to the specifications of the suppliers.

Cloning and expression of a phytase from *Peniophora lycii* CBS No. 686.96Deposited organisms:

The strain of *Peniophora lycii* has been deposited according to the Budapest Treaty
5 on the International Recognition of the Deposit of Microorganisms for the Purposes of
Patent Procedure at the Centraalbureau voor Schimmelcultures, P.O. Box 273, 3740
AG Baarn, The Netherlands, (CBS).

Deposit date : 4th of December 96
Depositor's ref. : NN006113
10 CBS No. : *Peniophora lycii* CBS No. 686.96

Still further, the expression plasmid (shuttle vector) pYES 2.0 comprising the full
length cDNA sequence encoding this phytase has been transformed into a strain of
the *Escherichia coli* which was deposited according to the Budapest Treaty on the
15 International Recognition of the Deposit of Microorganisms for the Purposes of Patent
Procedure at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.,
Masheroeder Weg 1b, D-38124 Braunschweig, Germany, (DSM).

Deposit date : 2nd of December 96
Depositor's ref. : NN 049282
20 DSM No. : *Escherichia coli* DSM No. 11312

Other strains:

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (van den Hazel,
H.B; Kielland-Brandt, M.C.; Winther, J.R. in Eur. J. Biochem., 207, 277-283, 1992;
25 (MATa; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1::LEU2;
cir+).

E. coli strain: DH10B (Life Technologies)

Plasmids:

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

5

pYES 2.0 (Invitrogen)

pA2phy2 (See example 1)

10 Expression cloning in yeast

Expression cloning in yeast was done as described by H. Dalboege et al. (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as reference. All individual steps of Extraction of total RNA, cDNA synthesis, Mung bean nuclease treatment, Blunt-ending with T4 DNA
15 polymerase, and Construction of libraries was done according to the references mentioned above.

Fermentation procedure of *Peniophora lycii* CBS No. 686.96 for mRNA isolation:

Peniophora lycii CBS 686.96 was inoculated from a plate with outgrown mycelium
20 into a shake flask containing 100 ml medium B (soya 30 g/l, malto dextrin 15 g/l, bacto peptone 5 g/l, pluronic 0.2 g/l). The culture was incubated stationary at 26°C for 15 days. The resulting culture broth was filtered through miracloth and the mycelium was frozen down in liquid nitrogen.

25 mRNA was isolated from mycelium from this culture as described in (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953).

Extraction of total RNA was performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)⁺RNA was
30 carried out by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 94/14953.

cDNA synthesis:

Double-stranded cDNA was synthesized from 5 mg poly(A)⁺ RNA by the RNase H method (Gubler and Hoffman (1983) Gene 25:263-269, Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). The poly(A)⁺ RNA (5 µg in 5 µl of DEPC-treated water) was heated at 70°C for 8 min. in a pre-siliconized, RNase-free Eppendorph tube, quenched on ice and combined in a final volume of 50 µl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, Bethesda Research Laboratories) containing 1 mM of dATP, dGTP and dTTP and 0.5 mM 5-methyl-dCTP (Pharmacia), 40 units human placental ribonuclease inhibitor (RNasin, Promega), 1.45 µg of oligo(dT)₁₈-Not I primer (Pharmacia) and 1000 units SuperScript II RNase H reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was synthesized by incubating the reaction mixture at 45°C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gelfiltrated through a MicroSpin S-400 HR (Pharmacia) spin column according to the manufacturer's instructions.

After the gelfiltration, the hybrids were diluted in 250 µl second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM MgCl₂, 10 mM (NH₄)₂SO₄, 0.16 mM bNAD⁺) containing 200 µl of each dNTP, 60 units *E. coli* DNA polymerase I (Pharmacia), 5.25 units RNase H (Promega) and 15 units *E. coli* DNA ligase (Boehringer Mannheim). Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours and additional 15 min. at 25°C. The reaction was stopped by addition of EDTA to a final concentration of 20 mM followed by phenol and chloroform extractions.

Mung bean nuclease treatment:

The double-stranded cDNA was precipitated at -20°C for 12 hours by addition of 2 vols 96% EtOH, 0.2 vol 10 M NH₄Ac, recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 µl Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM ZnSO₄, 0.35 mM DTT, 2% glycerol) containing 25 units

Mung bean nuclease (Pharmacia). The single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 min., followed by addition of 70 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction and precipitation with 2 vols of 96% EtOH and 0.1 vol 3 M NaAc, pH 5.2 on ice for 30 min.

5

Blunt-ending with T4 DNA polymerase:

The double-stranded cDNAs were recovered by centrifugation and blunt-ended in 30 ml T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM of each dNTP and 5 units T4 DNA polymerase (New England Biolabs) by incubating the reaction mixture at 16°C for 1 hour. The reaction was stopped by addition of EDTA to a final concentration of 20 mM, followed by phenol and chloroform extractions, and precipitation for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

15 Adaptor ligation, Not I digestion and size selection:

After the fill-in reaction the cDNAs were recovered by centrifugation, washed in 70% EtOH and dried. The cDNA pellet was resuspended in 25 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP) containing 2.5 µg non-palindromic BstXI adaptors (Invitrogen) and 30 units T4 ligase (Promega) and incubated at 16°C for 12 hours. The reaction was stopped by heating at 65°C for 20 min. and then cooling on ice for 5 min. The adapted cDNA was digested with Not I restriction enzyme by addition of 20 µl water, 5 µl 10x Not I restriction enzyme buffer (New England Biolabs) and 50 units Not I (New England Biolabs), followed by incubation for 2.5 hours at 37°C. The reaction was stopped by heating at 65°C for 10 min. The cDNAs were size-fractionated by gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC) in 1x TBE to separate unligated adaptors and small cDNAs. The cDNA was size-selected with a cut-off at 0.7 kb and rescued from the gel by use of b-Agarase (New England Biolabs) according to the manufacturer's instructions and precipitated for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Construction of libraries:

The directional, size-selected cDNA was recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The cDNAs were desalted by gel filtration through a MicroSpin S-300 HR (Pharmacia) spin column according to the manufacturer's instructions. Three test ligations were carried out in 10 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP) containing 5 µl double-stranded cDNA (reaction tubes #1 and #2), 15 units T4 ligase (Promega) and 30 ng (tube #1), 40 ng (tube #2) and 40 ng (tube #3, the vector background control) of BstXI-NotI cleaved pYES 2.0 vector. The ligation reactions were performed by incubation at 16°C for 12 hours, heating at 70°C for 20 min. and addition of 10 µl water to each tube. 1 µl of each ligation mixture was electroporated into 40 µl electrocompetent *E. coli* DH10B cells (Bethesda research Laboratories) as described (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). Using the optimal conditions a library was established in *E. coli* consisting of pools. Each pool was made by spreading transformed *E. coli* on LB+ampicillin agar plates giving 15.000-30.000 colonies/plate after incubation at 37°C for 24 hours. 20 ml LB+ampicillin was added to the plate and the cells were suspended herein. The cell suspension was shaken in a 50 ml tube for 1 hour at 37°C. Plasmid DNA was isolated from the cells according to the manufacturer's instructions using QIAGEN plasmid kit and stored at -20°C.

1 µl aliquots of purified plasmid DNA (100 ng/ml) from individual pools were transformed into *S. cerevisiae* W3124 by electroporation (Becker and Guarante (1991) Methods Enzymol. 194:182-187) and the transformants were plated on SC agar containing 2% glucose and incubated at 30°C.

Identification of positive colonies:

After 3-5 days of growth, the agar plates were replica plated onto a set of the phytate replication plates, and incubated for 3-5 days at 30°C. 1% LSB-agarose containing 0.2M CaCl₂ is poured over the plates and after 1-4 days the phytase positive colonies are identified as colonies surrounded by a clearing zone.

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the phytase-producing colonies identified.

5

Isolation of a cDNA gene for expression in *Aspergillus*:

A phytase-producing yeast colony was inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

10

DNA was isolated according to WO 94/14953 and dissolved in 50 ml water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis.

- 15 The cDNA insert was excised using the restriction enzymes Hind III and Xba I and ligated into the *Aspergillus* expression vector pHD414 resulting in the plasmid pA2phy2.

- 20 The cDNA inset of Qiagen purified plasmid DNA of pA2phy2 (Qiagen, USA) was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and synthetic oligonucleotide primers using an Applied Biosystems ABI PRISM™ 377 DNA Sequencer according to the manufacturers instructions.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

- 25 Protoplasts are prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12.

- 100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM CaCl₂). Protoplasts are
30 mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid) (Tove Christensen et al. Bio/Technology, pp 1419-1422 vol.6, Dec. 1988). The mixture is left at room

temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM CaCl_2 and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is
5 resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a
10 single colony after the second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the *A. oryzae* transformants are inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant is removed.

15

The phytase activity is identified by applying 20 μl supernatant to 4 mm diameter holes punched out in 1% LSB-agarose plates containing 0.1M Sodiumacetate pH 4.5 and 0.1% Inositol hexaphosphoric acid. The plates are left over night at 37°C. A buffer consisting of 0.1M CaCl_2 and 0.2M Sodium acetate pH 4.5 is poured over the
20 plates and the plates are left at room temperature for 1h. Phytase activity is then identified as a clear zone.

Fed batch fermentation:

Fed batch fermentation was performed in a medium comprising maltodextrin as a
25 carbon source, urea as a nitrogen source and yeast extract. The fed batch fermentation was performed by inoculating a shake flask culture of *A. oryzae* host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 7.0 and 34°C the continuous supply of additional carbon and nitrogen sources were initiated. The carbon source was kept as the limiting
30 factor and it was secured that oxygen was present in excess. The fed batch cultivation was continued for 4 days.

Isolation of the DNA sequence:

The phytase encoding part of the DNA sequence can be obtained from the deposited organism *Escherichia coli* DSM 11312 by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

Cloning and expression was done by using the Expression cloning in yeast technique as described above.

10

mRNA was isolated from *Peniophora lycii*, CBS No. 686.96, grown as described above.

Mycelia were harvested after 15 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *Peniophora lycii*, CBS No. 686.96, consisting of approx. 9×10^5 individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Phytase-positive colonies were identified and isolated as described above and inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm. DNA was isolated according to WO 94/14953 and dissolved in 50 µl water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and the DNA sequence of the cDNA encoding the phytase was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and synthetic oligonucleotide primers using an Applied Biosystems ABI PRISM™ 377 DNA Sequencer according to the manufacturers instructions.

The cDNA is obtainable from the plasmid in DSM 11312.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the phytase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the phytase gene was purified.

5 The gene was subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmid pA2phy2.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

10

Test of *A. oryzae* transformants

Each of the transformants were tested for enzyme activity as described above. Some of the transformants had phytase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the phytase

15 in *Aspergillus oryzae*.

EXAMPLE 2

Purification of the phytase from *Peniophora lycii* expressed in *Aspergillus oryzae*

20 The *Peniophora lycii* phytase was expressed in and excreted from *Aspergillus oryzae* IFO 4177.

Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear

25 solution. The filtrate was concentrated by ultrafiltration on 3kDa cut-off polyethersulphone membranes followed by diafiltration with distilled water to reduce the conductivity. The pH of the concentrated enzyme was adjusted to pH 7.5. The conductivity of the concentrated enzyme was 1.2 mS/cm.

30 The phytase was applied to a Q-sepharose FF column equilibrated in 20mM Tris/CH₃COOH, pH 7.5 and the enzyme was eluted with an increasing linear NaCl

gradient (0 → 0.5M). The phytase activity eluted as a single peak. This peak was pooled and $(\text{NH}_4)_2\text{SO}_4$ was added to 1.5M final concentration. A Phenyl Toyopearl 650S column was equilibrated in 1.5M $(\text{NH}_4)_2\text{SO}_4$, 10mM succinic acid/NaOH, pH 6.0 and the phytase was applied to this column and eluted with a decreasing linear
5 $(\text{NH}_4)_2\text{SO}_4$ gradient (1.5 → 0M). Phytase containing fractions were pooled and the buffer was exchanged for 20mM Tris/ CH_3COOH , pH 7.5 on a Sephadex G25 column. The G25 filtrate was applied to a Q-sepharose FF column equilibrated in 20mM Tris/ CH_3COOH , pH 7.5. After washing the column extensively with the equilibration buffer, the phytase was eluted with an increasing linear NaCl gradient (0 → 0.5M).
10 The phytase activity was pooled and the buffer was exchanged for 20mM Tris/ CH_3COOH , pH 7.5 by dialysis. The dialysed phytase was applied to a SOURCE 30Q column equilibrated in 20mM Tris/ CH_3COOH , pH 7.5. After washing the column thoroughly with the equilibration buffer a phytase was eluted with an increasing linear NaCl gradient (0 → 0.3M). Fractions from the SOURCE 30Q column were analyzed
15 by SDS-PAGE and pure phytase fractions were pooled.

The phytase activity is measured using the following assay:

10 μl diluted enzyme samples (diluted in 0.1 M sodium acetate, 0.01 % Tween20, pH 5.5) were added into 250 μl 5 mM sodium phytate (Sigma) in 0.1 M sodium acetate, 0.01 % Tween20, pH 5.5 (pH adjusted after dissolving the sodium phytate; the sub-
20 strate was preheated) and incubated for 30 minutes at 37°C. The reaction was stopped by adding 250 μl 10 % TCA and free phosphate was measured by adding 500 μl 7.3 g FeSO_4 in 100 ml molybdate reagent (2.5 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 8 ml H_2SO_4 diluted to 250 ml). The absorbance at 750 nm was measured on 200 μl sam-
25 ples in 96 well microtiter plates. Substrate and enzyme blanks were included. A phosphate standard curve was also included (0-2 mM phosphate). 1 FYT equals the amount of enzyme that releases 1 μmol phosphate/min at the given conditions.

Example 3

Determination of the phytase specificity by ^1H NMR spectroscopy

The hydrolysis of phytic acid (PA) catalyzed by the *Peniophora* phytase and by a commercial *Aspergillus niger* phytase (Phytase Novo®) was investigated (27 mM
5 phytate, 1 FYT/ml, pH 5.5 and 3.5, and 27°C) by ^1H NMR profiling the product mixture in the course of 24 hours.

In the following $(\text{Ins}(p,q,r,\dots))\text{P}_n$ denotes *myo*-inositol carrying in total n phosphate groups attached to positions p, q, r, \dots . For convenience $\text{Ins}(1,2,3,4,5,6)\text{P}_6$ (phytic acid)
10 is abbreviated PA. Please refer, however, to the section "Nomenclature and position specificity of phytases" in the general part of this application.

The technique provide specific information about initial points of attack by the enzyme on the PA molecule, as well as information about the identity of the end product. On
15 the other side the evolving patterns of peaks reflecting the composition of the intermediate product mixtures, provide a qualitative measure, a finger print, suitable for identification of similarities and differences between individual enzymes.

NMR, like most other analytical methods, can distinguish between stereo-isomers
20 which are not mirror images (diastereomers), but not between a set of isomers, which are mirror-images (enantiomers), since they exhibit identical NMR spectra.

Thus, $\text{Ins}(1,2,4,5,6)\text{P}_5$ (3-phosphate removed) exhibits a NMR spectrum different from $\text{Ins}(1,2,3,4,5)\text{P}_6$ (6-phosphate removed) because the isomers are diastereo-
25 mers.

However, the NMR spectra of $\text{Ins}(1,2,4,5,6)\text{P}_5$ (3-phosphate removed) and $\text{Ins}(2,3,4,5,6)\text{P}_6$ (1-phosphate removed) are identical because the isomers are enantiomers. The same holds for the pair $\text{Ins}(1,2,3,4,5)\text{P}_6$ (6-phosphate removed) and
30 $\text{Ins}(1,2,3,5,6)\text{P}_6$ (4-phosphate removed).

Thus, by NMR it is not possible to distinguish between a 3- and a 1-phytase, and it is not possible to distinguish between a 6- and a 4-phytase (or a L-3-/D-3- and a L-6-/D-6-phytase, respectively, using the lowest-locant rule).

- 5 Biased by the description of 3- and 6-phytases in the literature, we have used the terms 3- and 6-phytases for our enzymes, but, though unlikely, we do not actually know if we have 1- and a 4-phytases instead.

Experimental.

- 10 NMR spectra were recorded at 300 K (27°C) on a Bruker DRX400 instrument equipped with a 5 mm selective inverse probe head. 16 scans preceded by 4 dummy scans were accumulated using a sweep width of 2003 Hz (5 ppm) covered by 8 K data points. Attenuation of the residual HOD resonance was achieved by a 3 seconds presaturation period. The spectra were referenced to the HOD signal (δ 4.70).

15

- PA samples for NMR analysis were prepared as follows: PA (100 mg, Phytic acid dipotassium salt, Sigma P-5681) was dissolved in deionized water (4.0 ml) and pH adjusted to 5.5 or 3.5 by addition of aqueous NaOH (4 N). Deionized water was added (ad 5 ml) and 1 ml portions, each corresponding to 20 mg of phytic acid, were
20 transferred to screw-cap vials and the solvent evaporated (vacuum centrifuge). The dry samples were dissolved in deuterium oxide (2 ml, Merck 99.5% D) and again evaporated to dryness (stored at -18°C until use).

- For NMR analysis one 20 mg phytic acid sample was dissolved in deuterium oxide
25 (1.0 ml, Merck 99.95% D). The solution was transferred to a NMR tube and the ^1H NMR spectrum recorded. Enzyme solution (1 FYT, dissolved in/diluted, as appropriate, with deuterium oxide) was added followed by thorough mixing (1 minute). ^1H NMR spectra were recorded immediately after addition of enzyme ($t=0$), then after 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 195, 210 minutes (= 30 3.5 hours), 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 11.5, 13.5, 15.5, 17.5, 19.5, 21.5, and 23.5 hours. The pH in the NMR tube was measured. Additional spectra were acquired af-

ter 48 and 120 hours (5 days), where a portion of substrate (PA, 6 mg) was added to probe if the enzyme retained its catalytic activity.

By means of 2D NMR analysis of inositol phosphate mixtures obtained by partial digestion of PA, in conjunction with published NMR data (Scholz, P.; Bergmann, G., and Mayr, G.W.: *Methods in Inositide Research* (Ed. Irvine, R.F.), pp. 65-82, Raven Press, Ltd., New York (1990)), characteristic ^1H NMR signals attributable to $\text{Ins}(1,2,3,4,5,6)\text{P}_6$ (PA), $\text{Ins}(1,2,4,5,6)\text{P}_5$, $\text{Ins}(1,2,3,4,5)\text{P}_5$, $\text{Ins}(1,2,5,6)\text{P}_4$, $\text{Ins}(1,2,6)\text{P}_3$, $\text{Ins}(1,2)\text{P}_2$, and $\text{Ins}(2)\text{P}$, were identified and permitted relative quantification of these species during the course of the reaction.

At pH 5.5 as well as 3.5 the *Aspergillus* phytase attacks with a high degree of selectivity PA in the 3-position, whereas the *Peniophora* phytase at pH 5.5 with a high degree of selectivity attacks PA in the 6-position.

Accordingly, the *Aspergillus* phytase prove to be an essentially clean 3-phytase, whereas the *Peniophora* phytase at pH 5.5 appear to be an essentially clean 6-phytase.

The exact configuration of myo-inositol tetrakisphosphate produced by partial hydrolysis of phytic acid with the *Peniophora* phytase could be determined as outlined below, also allowing us to conclude whether the *Peniophora* phytase is a D-, L- or a D/L-6-phytase.

Other ways of determining the exact specificity is by determining optical rotation (Johnson, L. F.; Tate, M. E. Ann. N. Y. Acad. Sci. 1969, 165, 526-532; Irving, G. C. J.; Cosgrove, D. J. J. Bacteriol. 1972, 112, 434-438) or using a chiral HPLC column.

5

1. HPLC-isolation of myo-inositol tetrakisphosphate produced by partial degradation of phytic acid with the Peniophora phytase. Desalting (ion exchange, dialysis, (2), (4) and (9) and references herein)
2. NMR analysis to check purity (i), determine whether several diastereomer tetrakisphosphates are produced (ii), and determine which of these are produced (iii)
3. Synthesis of relevant polyols using reduction by boronhydrid (BH) of the corresponding carbonhydrates (10)
4. Disintegration using periodate, reduction by boronhydrid and dephosphorylation following (2). Identification of polyol using HPLC
- 15 5. Oxidation of polyol using L-iditol dehydrogenase and final identification of carbonhydrid using HPLC.

References:

- (2) Van der Kaay et al, Biochem. J., 312 (1995), 907-910
- (4) Irving et al, J. Bacteriology, 112 (1972), 434-438
- 20 (9) Stevens, L.R. in "Methods in Inositide Research" (Irvine, R.F. Ed.), 9-30 (1990), Raven Press, Ltd., New York.
- (10) Stephens, L. et al, Biochem. J., 249 (1988), 271-282

EXAMPLE 4

25 *In vivo* determination of the synergistic effect of 3- and 6-phytases in broilers

Experimental setup

Diet	3-Phytase	6-Phytase
30 Normal/Low in Phosphorus	FYT/kg	FYT/kg
1 Normal-P	0	0
2 Low-P	0	0

	3 Low-P	0	500
	4 Low-P	125	375
	5 Low-P	250	250
	6 Low-P	375	125
5	7 Low-P	500	0

Phytases

The 3-phytase is Phytase Novo™ which is commercially available from Novo Nordisk A/S, Denmark

10

The 6-phytase is a phytase originating from *Peniophora lycii* CBS 686.96 (obtained and purified as described in Examples 1 and 2).

Experimental procedure

15

Animals

Male broiler chickens are housed on deep litter under conventional conditions from 1 to 14 days of age and fed a commercial starter diet. On day 14 the birds are weighed individually and birds with low or high body weights are discarded. Groups of 4 birds
20 are then assigned randomly to 35 digestibility cages and for each treatment 5 replicates are used.

Diets

The Ca level is 0.65 % and the P-level in the negative control 0.41 %. The P-level in the
25 normal diet is 0.41 % + 0.18 % mineral P giving a total of 0.59 % P.

The approximate dietary composition is displayed in Table 1.

Table 1

30

Approximate composition of the experimental diets

Ingredients		Basal diet ^A	Test diet ^A
	Maize	56.13	-
35	Maize	-	55.86
	Soybean oil meal	32.45	30.83
	Animal renderers fat	6.00	7.96

34

	Soybean oil	1.04	1.00
	Di-calciumphosphate x 2H ₂ O	2.24	-
	CaCO ₃	0.66	-
	NaCl	0.35	0.34
5	DL-Methionine	0.13	0.13
	Vitamin and mineral premix	1.00	1.00
	Supplement ^B	-	2.90

^ABoth diets are supplemented with monensin (100 mg/kg) and avoparcin (10 mg/kg).

10 ^BSupplies variable amounts of CaCO₃, Di-calcium phosphate, heat treated wheat and phytase formulation.

15 *Balance trial*

The *in vivo* balance trial is conducted according to the European reference method which consists of a 7-day period of adaptation and a 4-day balance period. During the main balance period the birds are fed the respective diets at a level of 90 % of *ad libitum* intake and their excreta is collected quantitatively each day.

20

Chemical analyses

Samples of diets and freeze dried excreta are analysed for dry matter, ash, Kjeldahl nitrogen (crude protein, Nx6.25), lipids (for diets only), calcium (oxalate precipitation), phosphorus (phospho-vanadomolybdate colorimetry), and gross energy (adiabatic bomb calorimetry).

25

Samples

Dietary samples, approximately 500 g, are analyzed for in-feed enzymatic activity.

30 **EXAMPLE 5**

***In vitro* determination of release of phosphorous from phytate using 3- and 6-phytase in combination**

Inositol phosphate spectrum by Anion exchange HPLC and post-column Ferri /

Sulfosalicylic acid reaction

35

Phytate is the fully phosphorylated inositol with phosphates in position 1 to 6, which is de-phosphated by phytase.

The hydrolysis pattern of this reaction is described. Degradation of phytate(IP₆) to IP₅,
5 IP₄, IP₃, IP₂, IP and I(inositol) is therefore studied. In this example only the degree of phosphorylation is described, not the isomers; as the method used detect totals of a certain phosphorylation level.

After phytase incubation with phytate, the hydrolysis reaction is slowed down (or
10 stopped) by addition of 4 N HCl till pH is around 1.3. The resulting inositol phosphate spectrum in this reaction mixture is quantified by Anion ion-exchange chromatography with post column reaction. IP2 to IP6 are well identified.

(Ref: Anion-exchange high-performance liquid chromatography with post-column de-
15 tection for the analysis of phytic acid and other inositol phosphates. Rounds & Nielsen. Journal of Chromatography A, 653 (1993) 148-52).

Materials and Methods

Hydrolysis condition

20

Phytate concentration	6 mM
Phytase activity	0.18 FYT/mL
pH	5.0
Buffer, Acetate	100 mM
Temperature	37°C
Hydrolysis time	0 min. to 48 h
Stop reaction	70 mL 4M HCl/2mL

Reagents

Acetate buffer, 100 mM, pH 5.0

25 5.80 ml of Acetic acid in 500 mL of water

13.6 g Na-Acetate•3H₂O dissolved in 500 mL of water.

An aliquot of 352 mL Acetate, is mixed with 148 mL Acetic acid and diluted to 1000 mL with water, pH is checked with NaOH/HCl.

Phytate 12 mM

- 5 Na-phytate dissolved in Acetate buffer, pH 5.0.

Phytase

Phytase is diluted with Acetate buffer, pH 5.0 until 0.36 FYT/mL.

Experiments with phytase (0.18 FYT/mL incubation mixture) and reaction time from 0
10 to 48 h are being performed.

Enzyme types

Two phytases are being tested in this assay:

A 6-phytase originating from Peniophora CBS 686.96 (obtained and purified as de-
15 scribed in Examples 1 and 2).

A commercial 3-phytase originating from Aspergillus niger (Phytase Novo™).

3- and 6-Phytase mixtures

Enzyme mixtures of 3- and 6-specific phytase are prepared as: 25, 50 and 75% of
20 one of the specific phytases while the other is added up to 100%, 0.36 FYT/ml. Thus
all incubation mixtures have a total phytase activity of 0.18 FYT/ml.

Enzyme-substrate incubation

Incubation

25 Test tube with stirring bar, cross type, is placed in water bath at 37°C.

1 mL of phytate is added. 1 mL of phytase is added and the test tube with this incubation solution is kept stirred at 37°C.

Timing of incubation

The reaction times are: 0 min - 5 min - 10 min - 30 min - 1 - 3 - 24 and 48 hours.

Stop of incubation

The reaction is stopped by adding 70 mL 4 M HCl/tube to the incubation mixture, mixing and placing at room temperature.

Sampling of incubation mixture

- 5 Aliquots of the stopped incubation mixture are transferred to the HPLC sampler and analyzed without further dilution.

HPLC conditionsEluents

- A) 0.01 M 1-Methyl piperazin in Milli-Q water.
10 B) 0.01 M 1-Methyl piperazin in Milli-Q + 0.5 M NaNO₃ in Milli-Q.

Postcolumn eluent) 0.015 % FeCl₃ + 0.15 % Sulfosalicylic acid in Milli-Q

Gradient profile

- 15 Elution of inositol phosphates required a gradient. The conditions are:

Time	0.01 M 1-Methyl piperazin	0.01 M 1-Methyl piperazin + 0.5 M NaNO ₃
	% of flow	% of flow
0	100	0
30	25	75
31	0	100
32	0	100
33	100	0
40	100	0

Column

An anion exchange column, Mono Q® 5/5 from Pharmacia is used.

Reaction coil

For the postcolumn Fe-complex reaction, a flow of 2 mL/min in a 1.60 m PEEK tube with ID. 0.762 mm is being used. The total reaction time is app. 22 seconds.

Detection

- 5 The decrease in absorbance at 500 nm, from phosphate complexing with iron in the post column reaction, is recorded as "positive" peaks by reversing polarity of the detector chart connections.

Experimental

- Five peaks are identified in each assay. They are inositol di-phosphate (IP₂), inositol
10 tri-phosphate (IP₃), inositol tetra-phosphate (IP₄), inositol penta-phosphate (IP₅) and Phytic acid (inositol hexa-phosphate, IP₆). The peaks are named as follows:

Peak No	Elution time, min	Compound
1	8.5	IP2
2	12.1	IP3
3	16.5	IP4
4	22.4	IP5
5	26.7	IP6

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>19</u> , line <u>8 - 10</u> .	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 4 December 1996	Accession Number <i>Peniophora lycii</i> CBS No. 686.96
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional <input type="checkbox"/>	
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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A. The indications made below relate to the microorganism referred to in the description on page <u>19</u> , line <u>18 - 20</u> .	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY	
Date of deposit 2 December 1996	Accession Number <i>Escherichia coli</i> DSM No. 11312
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional <input type="checkbox"/>	
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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CLAIMS

1. A composition comprising at least two phytases of different position specificity.
- 5 2. The composition according to claim 1 which is a food or an animal feed.
3. The composition according to claim 1 which is a food additive or an animal feed additive.
- 10 4. The composition according to any of claims 1-3 which comprises a 3-phytase and a 6-phytase.
5. A process for preparing a feed or a food, which comprises the step of adding at least two phytases of different position specificity or a composition according to
15 claim 3 to feed or food components.
6. A process for preparing a food additive or an animal feed additive, which comprises the step of adding at least two phytases of different position specificity or a composition according to claim 1 to additive components.
20
7. A process for reducing phytate levels in animal manure, which process comprises feeding an animal with an effective amount of the feed according to claim 2 or the feed obtainable according to claim 5.
- 25 8. Use of at least two phytases of different position specificity for liberating phosphorous from a phytase substrate.
9. Use of at least two phytases of different position specificity for improving the food or feed utilization.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00586

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/16, A23K 1/165, A23L 1/03
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A23K, A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, FOODSCI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9528850 A1 (NOVO NORDISK A/S), 2 November 1995 (02.11.95), see page 9, line 26 - page 10, line 5 --	1-9
A	EP 0619369 A1 (AVEVE N.V.), 12 October 1994 (12.10.94) -- -----	1-9

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

- * Special categories of cited documents:
- *A* document defining the general state of the art which is not considered to be of particular relevance
 - *B* earlier document but published on or after the international filing date
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 - *O* document referring to an oral disclosure, use, exhibition or other means
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 - *Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search

7 April 1998

Date of mailing of the international search report

20 -04- 1998

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Yvonne Siösteen

Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/03/98

International application No.

PCT/DK 97/00586

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